Studies of Glyoxalase I Substituted with Manganese in its Active Site *

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Glyoxalase I from mammalian sources is a dimeric protein composed of two identical or very similar subunits (see Refs. 1 and 2 for review). The native enzyme contains two Zn atoms which can be replaced by other bivalent metal ions such as Mg^{2+}, Mn^{2+} and Co^{2+} (see Ref. 3). Substitutions of Zn in the active site of glyoxalase I with the above metals give catalytically active enzymes with specific activities >60% of that of the native Zn protein.[3] The Mn enzyme is suitable for studies of the active site by means of proton magnetic resonance using the relaxation rates of water protons interacting with the paramagnetic Mn atom. Such studies may eventually lead to a stereochemical model of the substrate bound to the active site (see Ref. 4). The present study shows how titration of the enzyme with the strong competitive inhibitor *S-p*-bromobenzylglutathione can be monitored by NMR spectroscopy and that the data obtained are consistent with the results of experiments in which binding is measured by means of the intrinsic tryptophan fluorescence.

Experimental. Glyoxalase I from human erythrocytes was purified as previously described and a mixture of the three isoenzymes was used.[5] Enzymatic activity was measured using methylglyoxal as the 2-oxoaldehyde substrate.[1,6] Protein concentrations were determined by a microbiuret method using serum albumin as a standard.[7] Apoenzyme was prepared by dialyzing glyoxalase I at 4 °C against 1 mM EDTA in 50 mM imidazole/HCl buffer (pH 6.8) containing 10% methanol.[3] Mn glyoxalase I was prepared by addition of an about 20-fold molar excess of $MnCl_2$ over the molar concentration of apoenzyme subunits. Excess of Mn^{2+} was removed by dialysis at 4 °C against 10 mM Tris/HCl buffer (pH 7.8) containing 10% methanol.[3] All buffer solutions were pretreated with Chelex 100 (BioRad) to remove contaminating metal ions.[8] *S-p*-Bromobenzylglutathione was synthesized by method A of Vince *et al.*[9] All other chemicals were of the highest purity available commercially. Intrinsic fluorescence of glyoxalase I and its quenching by the competitive inhibitor *S-p*-bromobenzylglutathione were measured at 22 °C on a Jasco FP-4 spectrofluorimeter in a rectangular 1-cm quartz cell. The wavelengths for excitation and emission were 280 and 344 nm, respectively. Proton relaxation was measured at 20 °C on a Varian model XL-100A FT-NMR spectrometer operating at 100 MHz with a deuterium internal lock. The sample solutions in 5 mm (outer diameter) tubes contained about 10% D_2O. Longitudinal relaxation times (T_1) were measured by a $180°-\tau-90°$ sequence for 10 different delay times. Computation of T_1 was made by a linear least-squares fit. The experimentally determined relaxation rate ($1/T_{1.obs}$) was corrected for the diamagnetic contribution ($1/T_{1.diam}$) in order to obtain the paramagnetic relaxation rate:

$$1/T_{1.p} = 1/T_{1.obs} - 1/T_{1.diam}$$

Results and discussion. The apoenzyme reactivated with Mn^{2+} contained 2 Mn per dimeric enzyme molecule and had 67% of the catalytic activity of the native enzyme.[3] The Mn enzyme, like native glyoxalase I, has intrinsic fluorescence assigned to tryptophan residues.[10] The fluores-

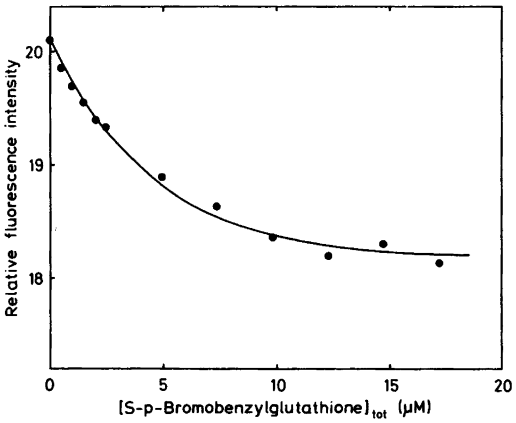

Fig. 1. Titration of the intrinsic tryptophan fluorescence of Mn glyoxalase I with increasing concentration of *S-p*-bromobenzylglutathione. The dots show the experimental points and the line the curve fitted by nonlinear regression analysis (dissociation constant = 1.6 ± 0.3 μM; binding stoichiometry 2 mol of ligand per mol of enzyme). The enzyme concentration was 0.39 μM in 10 mM Tris HCl buffer (pH 7.8) containing 10% methanol. The temperature was 22 °C.

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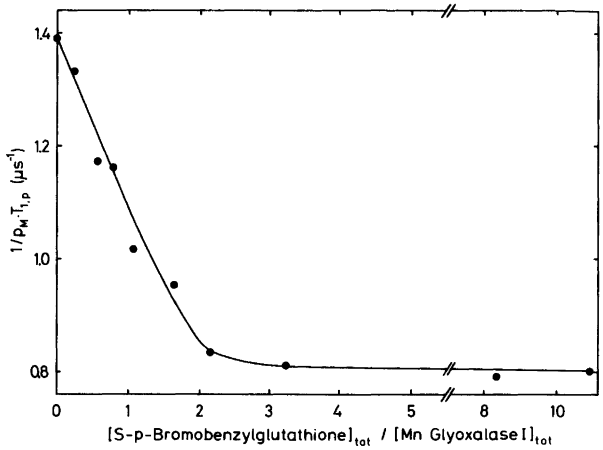

Fig. 2. Titration of Mn glyoxalase I with increasing concentration of *S-p*-bromobenzylglutathione monitored by the paramagnetic proton relaxation rate of water ($1/p_M T_{1,p}$). The normalizing factor, p_M, is [protein-bound Mn^{2+}]/[H_2O]. The dots show the experimental points and the line the curve computed by use of the binding parameters estimated in Fig. 1. The enzyme concentration was 79 μM in 10 mM Tris HCl buffer (pH 7.8) containing 10% methanol. The temperature was 20 °C.

cence is quenched by about 10% after addition of excess of the competitive inhibitor *S-p*-bromobenzylglutathione. Fig. 1 shows a titration curve obtained by adding increasing amounts of the ligand to the Mn enzyme. From this titration a dissociation constant of 1.6 ± 0.3 μM for the binding of the ligand to the Mn enzyme was calculated by nonlinear regression analysis.[10] Native enzyme has a dissociation constant of 0.15 μM.[10]

The Mn-containing enzyme showed an enhanced nuclear relaxation rate of water protons. Addition of excess of *S-p*-bromobenzylglutathione decreased the enhancement factor from 3.4 to 2.1. Fig. 2 shows the results of a titration with varying additions of the glutathione derivative. The solid line is the theoretical curve computed by use of the estimated dissociation constant of 1.6 μM (*cf.* Fig. 1) and a binding stoichiometry of 2 mol/mol (*cf.* Ref. 10). The agreement between the titrations made by the two different methods is very satisfactory. The proton relaxation measurements were made at high enzyme concentrations and give the best estimate of the binding stoichiometry (1.7 ± 0.3 mol/mol) whereas the fluorescence measurements were made at lower enzyme concentrations and yield the best estimate of the dissociation constant (1.6 ± 0.3 μM).

The dissociation constant for binding of *S-p*-bromobenzylglutathione to Mn glyoxalase I is in good agreement with the inhibition constant (1.7 ± 0.2 μM) determined by kinetic experiments carried out under steady-state conditions and computed under the assumption of linear competitive inhibition.[10] This agreement suggests that the different methods all monitor binding of the ligand to the active site of glyoxalase I. Consequently, the spectroscopic methods present the possibility of gauging distances between the metal in the active site and ligands bound in the vicinity of the metal.

The results of the proton relaxation measurements make possible conclusions about the coordination scheme of enzyme, metal and ligand.[11] The finding that the enhancement of the proton relaxation rate of water decreased from 3.4 to 2.1 when *S-p*-bromobenzylglutathione is bound to glyoxalase I is consistent with the "metal bridge" coordination, *i.e.* the ligand is bound to the enzyme *via* the metal.[11] The details of the interaction of *S-p*-bromobenzylglutathione and other glutathione derivatives with Mn in the active site of glyoxalase I is under investigation.

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